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Effects of Passive Antibody on Early Pathogenesis of Marek's Disease

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The effects of passive Marek's disease virus (MDV) antibody were characterized in three experiments in which 1- or 2-day-old PDRC chickens were inoculated with cell-associated MDV. Antibody acquired naturally from the dam, or injected prior to, or at the time of, virus inoculation, failed to prevent infection, but the incidence and severity of the acute necrotizing disease were reduced. Also, the number of tissues with viral antigen (fluorescent antibody test) and the amount of antigen in positive tissues was lowered, and fewer infected cells in spleen (in vitro assay) were detected in antibody-positive chicks. Serum containing MDV antibody was efficacious when injected prior to infection and had some protective effect when first given at day 4 but not at day 7 after virus inoculation. Normal antibody-free serum was ineffective, and chickens with naturally acquired (maternal) antibody against turkey herpesvirus (a virus antigenically related to MDV) were not protected against MDV infection.

The effects of Marek's disease (MD) herpesvirus infection on the immune response of chickens and, conversely, the effects of an immune response on the pathogenesis of MD are of considerable interest. Organs associated with the immunological response, e.g., the bursa of Fabricius, the thymus, and the bone marrow, are sites of early virus replication (3, 20, 26, 28) which can result in necrosis, atrophy, and aplasia of the organs (14, 15, 21). These were most pronounced (14) in chickens lacking maternal antibody when exposed to virus. The loss of lymphoid cells may have accounted for the decreased or delayed antibody response, lowered resistance to other diseases, and delayed homograft rejection, all of which have been associated with MD (2, 16, 19, 22).

Passive antibody blocks the early destructive lesions caused by MD virus (MDV), delays the development of humoral MD antibody, and renders chickens more resistant to tumorigenesis (1, 7; Calnek, Proc. 5th Int. Symp. on Comp. Leukemia Res., *in press;* Spencer and Robertson, Amer. J. Vet. Res., *in press*). This formed the basis of attempts to reduce the incidence of MD in commercial chickens by hyperimmunization of the dams (1). Since humoral antibody is protective against the pathological consequences of infection with MDV, a rapid humoral antibody response on the part of the host might have considerable survival value. Several reports showed that birds differing in susceptibility to MD also differed in immunological responsiveness (9, 17, 27; Calnek, Proc. Int. Symp.: Oncogenesis Herpesviruses Res., *in press*). Although no association could be made between precipitin and resistance, neutralizing antibody and resistance seemed correlated (Calnek, Proc. Int. Symp.: Oncogenesis Herpesviruses Res., *in press*).

Because of these observations, we wished to further characterize the effects of passive antibody on the infectivity and pathogenesis of MD. In three experiments, chickens with and without passive antibody were exposed to virulent MDV and then examined for distribution and amount of viral antigen, virus, and pathological lesions.

MATERIALS AND METHODS

Parent flocks. Chickens were of the departmental PDRC strain (3; Calnek, Proc. Int. Symp.: Oncogenesis Herpesviruses Res., *in press*) of single-comb White Leghorns. Two flocks (A and C), raised in isolation, provided chickens free of MDV antibody. Sera, collected before and after progeny were obtained, were all negative in the agar gel precipitin (AGP) test (6) for antibody. Two parallel flocks (B and D) were raised on the main departmental farm where MDV is indigenous. Both had a high incidence of precipitin (88 to 100% positive in the AGP test), and virus neutralization tests on 11 sera from flock D were all positive.

A fifth parent flock (E), described elsewhere (Patrascu, Calnek, and Smith; Avian Dis., *in press*), was vaccinated with turkey herpesvirus (HVT) strain FC 126 (29), a virus antigenically related to MDV and used as a vaccine against MD in chickens (18). All of the dams and their progeny carried virus neutralization antibody against HVT.

Experimental chickens. All chickens for a given experiment were hatched together and subsequently held in the same wire-floored battery brooder in an isolation unit (experiments 2 and 3) or in Horsfall-type cages (experiment 1).

Virus inocula. The JM isolate of MD (24) was employed for all experiments. Four batches were used; they consisted of suspensions of gonadal tumor cells (JM-1, -2, -4) or buffy coat cells plus tumor cells (JM-3). All were stored as viable cell suspensions at −196 C by techniques described elsewhere (25). Titrations on chicken kidney cell cultures indicated that the doses per bird for JM-1, JM-2, and JM-3 were 240, 97, and 280 focus-forming units (FFU), respectively. JM-4 was not titrated. Chickens were inoculated intra-abdominally with 0.1-ml (experiments 1 and 2) or 0.25-ml quantities.

Sera for injections. One serum pool (No. 325), free of precipitin and neutralizing MDV antibody, was obtained from three flock B birds. There were two antibody-positive serum pools (No. 29, 415) obtained from flock C and D birds, respectively. MDV exposure for these flocks was natural and thus the antibody titers reflected natural levels. Virus neutralization and AGP tests were conducted as described elsewhere (Calnek, Proc. Int. Symp.: Oncogenesis Herpesviruses Res., *in press*). Both pools had virus neutralization antibody titers of 160 (reciprocal of highest dilution to cause a 50% reduction in virus titer); precipitin titers (reciprocal of highest dilution to give a line of precipitation) were 32 and 64 for the respective pools. Sera were injected at 1 ml per dose per chicken—0.5 ml intra-abdominally and 0.5 ml subcutaneously. Sera given the same day as virus inoculations were administered about 30 min earlier.

Chicken examinations. Chickens were examined for clinical signs (depression) and then killed. For experiments 1 and 2, histological examinations were made on hematoxylin and eosin stained tissue sections of a variety of organs. To aid in comparing groups, selected organs (bursa of Fabricius, thymus, kidney) were scored on a scale of 1 to 4 for the histopathology. For the bursa, a score of 1 was assigned when small areas of necrosis or some loss of lymphocytes from the medullae were observed. Marked necrosis, cysts, interfollicular connective tissue proliferation, and severe atrophy of the organ with no normal follicles were criteria for a score of 4, whereas intermediate degrees of pathological changes were scored 2 or 3. Scores for thymic changes varied from 1, for a rare isolated area of necrosis, to 4, for severe necrosis involving more than 50% of the tissue and marked atrophy. Kidneys with rare isolated lesions consisting of glomerulitis or focal necrosis or a rare focus of lymphoid cells, or both, were scored 1. Massive areas of tubular degeneration (dilatation in some cases) and many areas of tubular and glomerular necrosis, often accompanied by large or numerous lymphoid foci, or both, were scored 4. All normal tissues were scored 0.

For experiment 3, the bursa from each bird was removed and weighed, and both the bursa and thymus were scored for degree of gross atrophy (1 to 4 denoted organs about 75, 50, 25, or less than 25% the size of those in control chickens).

Fluorescent antibody (FA) tests for MDV antigen (3) were done on frozen tissue sections of the same organs examined histopathologically. Those from the skin, bursa, thymus, and kidney in experiments 1 and 2 were scored. Antigen in the skin was assessed by examining at least 10 feather follicles; scores of 1 to 4 denoted variation from a few positive cells in one follicle to many positive cells in all follicles. At least 50 bursal follicles and one cross section of a thymic lobe were examined. Scores of 1 to 4 indicated variation from rare isolated positive cells to many large areas in which most of the cells were positive. In a cross section of the anterior lobe of one kidney, a single focus of positive cells was scored as 1, two to five foci as 2, six to 20 foci as 3, and more than 20 foci as 4. All negative tissues were scored 0.

Virus isolation attempts. Spleen cells, dispersed by treatment with trypsin and ethylenediaminetetraacetic acid, were treated with dimethyl sulfoxide as a protectant (25) and were stored at −70 C for later assay (experiment 2) or were assayed fresh (experiment 3). Before assay, a portion of each suspension was treated with trypan blue and examined by microscope with the aid of a hemocytometer to determine the number of viable cells. At 6 to 7 days after inoculation of the spleen cells onto 24- or 48-hr chicken kidney cultures, MDV foci were counted and the number of FFU per million viable inoculum cells was determined.

RESULTS

Experiment 1. Nineteen antibody-free flock A chickens and ten antibody-positive flock C chickens were given JM-1 virus at 1 day of age. Ten chicks from each source were uninoculated controls. At 7, 10, 12, and 14 days, two chickens from each inoculated group were removed for examination. Two control chickens from each source were examined at 10 and 14 days. Experiment details and results of the examinations for clinical signs (depression, stunting) and for histopathological lesions and viral antigen in selected organs are presented in Table 1. The incidence and severity of pathological changes and the amount of viral antigen in tissues were markedly depressed in the antibody-positive chickens. The control chickens were free of lesions and antigen.

In addition to the birds killed, 10 of the infected, antibody-free chickens died between 10 and 17 days postinoculation. Lesions in those and in the affected sacrificed birds were primarily degenerative. Bursal and thymic changes and aplasia of bone marrow were essentially similar to those described by Purchase and Biggs (21) and Jakowski et al. (14). Chickens with severe

TABLE 1. *Effect of naturally acquired (maternal) MDV or HVT antibody or injected MDV antiserum on the pathological response, viral antigen, and infectivity of spleen cells in PDRC chickens at 7 to 14 (experiment 1) or 11 to 18 (experiment 2) days after inoculation with cell-associated MDV*[a]

Expt	Antibody status of chickens[b]		No. of birds exam	No. with clin signs	Avg lesion[c] score			Avg score in the FA test for antigen[d]				Virus isolations—spleen:	
	Type	Source			Bur	Thy	Kid	FFE	Bur	Thy	Kid	Positive/tested	FFU per 10^6 cells
1	None		8	6	3.5	3.1	2.3	0.3	2.9	1.9	2.3	ND	ND
	MDV	Natural	8	0	0.7	0	0.4	0	0	0	0	ND	ND
2	None		8	6	2.4	1.4	1.6	2.3	0.8	0.1	1.1	5/5	68
	MDV	Natural	6	1	1.7	0	0.5	0.2	0.2	0	0	0/3	0
	MDV	Injected: days −1, 0, 7	8	0	0.9	0	0.4	0.5	0	0	0	2/5	15
	MDV	Injected: days 0, 1, 7	6	0	0.5	0	0.3	0	0	0	0	1/3	<1
	HVT	Natural	6	1	2.2	1.0	1.2	1.2	0.7	0.3	0.8	1/3	5

[a] Chickens inoculated intraabdominally with MDV-infected tumor cells (JM-1 at 1 day of age for experiment 1, JM-2 at 2 days for experiment 2). Eight uninoculated controls for experiment 1, and four for experiment 2 were similarly examined and found negative in all respects.

Abbreviations: MDV, Marek's disease virus; HVT, turkey herpesvirus; exam, examined; clin., clinical; Bur, bursa of Fabricius; Thy, thymus; Kid, kidney; FFE, feather follicle epithelium; FA, fluorescent antibody; FFU, focus forming units; ND, not done.

[b] Injected: A total of 1 ml of MDV antiserum batch no. 29 was injected subcutaneously and intraabdominally on each of the days indicated (day 0 is the day MDV was injected).

[c] Lesions scored 1 to 4 depending on severity; normal organs scored 0.

[d] Amount of viral antigen compared on the basis of number of positive cells and intensity of reaction; positive tissues scored 1 to 4. Negative tissues scored 0.

bursal and thymic necrosis often had inflammatory lesions (edema, cellular infiltration, necrosis) in the skin and degenerative changes in the kidney. The latter were characterized by focal necrosis of tubular epithelium, as described by Fletcher et al. (10), plus glomerulitis or focal necrosis in glomeruli, or both, dilation of tubules, generalized degeneration of all parenchymal cells, and lymphoid cell infiltration around areas of focal necrosis. Intranuclear inclusion bodies were found in many of the cells comprising the necrotic foci. Grossly, kidneys were swollen and pale, and they contained an overabundance of urates. Other lesions often seen in the more severely affected birds included focal or generalized necrosis in the pancreas, proventriculus, liver, spleen, and heart. These lesions also were inhibited in the antibody-positive chickens. Nerve plexi and gonads were not sites of necrotic lesions, although lymphoid cell accumulations were occasionally seen in these tissues.

FA tests showed that the location of antigen usually matched the location of lesions and, in general, the amount of antigen correlated well with the severity of the lesions. However, some affected bursas, especially in birds with lesions of only moderate intensity (score of 2 or 3), had no detectable viral antigen.

The single, infected, antibody-free bird surviving at 28 days had lymphoid tumors characteristic of MD involving peripheral nerves and visceral organs. There was no mortality in the uninoculated control group or in the infected, antibody-positive group. One of the two surviving infected birds had gross MD lesions, but all 12 controls were negative at 28 days.

Experiment 2. The experimental design was similar to that for experiment 1 except that additional groups were included to assess the influence of injected MDV antibody and to determine if naturally acquired (maternal) HVT antibody could depress the effects of MDV infection. Also, spleen cells were assayed for infectivity. In this case, the inoculum was JM-2 and the chickens were from flocks B (no antibody), D (MDV antibody), and E (HVT antibody). Chickens were examined at 11, 14, and 18 days postinoculation. Details and results are recorded in Table 1. Every virus-inoculated chicken had evidence of infection by one or more criteria. However, as in experiment 1, naturally acquired MDV antibody reduced the average

lesion and FA test scores. In addition, injected antiserum was efficacious. HVT maternal antibody, on the other hand, was ineffective in preventing lesions. The nature and extent of pathological changes in affected birds were similar to those described for experiment 1 birds. Again, some birds with moderately severe bursal lesions did not have viral antigen in that organ. This was reflected in the lower average scores for FA tests than for histopathological lesions (Table 1).

Virus isolations were more frequent, and more virus was demonstrated in spleen cells from the antibody-free group than from any other group. Four uninoculated controls (two each from flocks B and D) were negative in all respects.

No birds died during the first 30 days postinoculation. At day 31, three remaining antibody-free chickens all had evidence of advanced Marek's disease: one was dead, another slightly depressed, and all three had gross leukotic tumors involving nerves or visceral organs, or both. All other birds were clinically normal, but one of three flock E (HVT antibody) chicks had splenomegaly and another had leukotic tumors in nerves and viscera. Two of three birds injected with MD antibody at days 0, 1, and 7 had some splenomegaly (no tumors), whereas three with natural maternal antibody (flock D) and four injected with antibody on days −1, 0, and 7 were free of gross lesions. Four uninoculated control birds were negative.

Experiment 3. Flocks B (antibody-free) and D (MDV antibody) progeny were inoculated with JM-3 or JM-4 virus at 2 days of age. Various groups of three chickens were also injected with antibody-free serum (No. 325) or antibody-positive serum (No. 29 or No. 415) on 2 successive days beginning 1 day before, or 4 or 7 days after, MDV inoculation. All chickens were killed at 10 days postinoculation. Bursal weights and scores for gross atrophy of bursas and thymuses were recorded, and virus isolations were attempted on spleen cells. The results are recorded in Table 2. Natural maternal antibody was again effective in preventing atrophic changes and in reducing the infectivity of spleen cells. In three instances, injected antiserum was found highly efficacious when injected before infection; a partial protection was afforded when administration was begun at 4 days after infection, but no effect was detected when it was delayed until 7 days after infection. Antibody-free serum was ineffective. The results of virus isolation attempts from spleen cells were somewhat erratic, but the

TABLE 2. *Effect of naturally acquired (maternal) antibody or injected antiserum on the pathological response and infectivity of spleen cells in PDRC chicks at 10 days after inoculation with cell-associated MDV*[a, b]

Virus inoculum	Maternal antibody	Serum injections[c]			Avg from three chickens per group			
		Batch no.	MDV Ab	Days injected	Bursa wt (mg)	Bursa lesion score[d]	Thymus[d] lesion score	Virus isolations from spleen (FFU/10^6 cells)
None	Yes	None			396	0	0	0
JM-3	No	None			83	3.2	3.7	105
	Yes	None			342	0	0	1
	No	415	Yes	−1, 0	294	0.7	0.7	1
		415	Yes	4, 5	172	1.7	2.0	12
		415	Yes	7, 8	110	2.7	3.7	25
	No	29	Yes	−1, 0	277	0.7	0	10
		29	Yes	4, 5	150	2.0	1.5	43
		29	Yes	7, 8	118	3.0	2.8	28
	No	325	No	−1, 0	149	2.2	3.0	18
JM-4	No	None			129	2.8	2.7	31
	No	415	Yes	−1, 0	286	0	0	5
		415	Yes	4, 5	172	1.7	2.3	3
		415	Yes	7, 8	139	3.0	3.3	42

[a] Experiment 3: Chickens inoculated intra-abdominally with MDV-infected tumor cells (JM-4) or tumor cells plus buffy coat cells (JM-3) at 2 days of age.

[b] Abbreviations: MDV, Marek's disease virus; Ab, antibody; FFU, focus-forming units.

[c] Injections: total of 1 ml of serum given intra-abdominally and subcutaneously on each of 2 successive days; day 0 is the day MDV was injected.

[d] Severity of gross pathology (atrophy) scored from 1 to 4. Normal organs scored 0.

number of positive attempts and the level of infection (FFU per 10^6 cells) was generally positively correlated with the extent of pathological change.

DISCUSSION

These studies confirmed the observations that early destructive lesions of hematopoietic tissues are largely prevented by maternal antibody (16). Further, although the data are meager, there is additional support for the observation (7) that the presence of passive antibody at the time of virus exposure can reduce the subsequent incidence of neoplastic lesions. Beyond these confirmatory findings, the nature of the antibody effect has been partly characterized.

The results of the FA tests for specific MDV antigen provide firm substantiation for the association between MDV infection and the early necrotizing pathology since, in most cases, the location and amount of viral antigen correlated well with the pathological lesions. There were exceptions. In many groups, particularly those which received the less virulent inocula (evidenced by lack of mortality or clinical signs, few gross lesions, and only moderately severe microscopic pathology), there were tissues with pathological lesions but no detectable viral antigen. Possible explanations include the following: (i) the pathology was due to an agent other than MDV, (ii) the pathology was due to MDV infection, but cell death occurred before sufficient amounts of the particular viral antigens detected by the FA conjugate were elaborated (or they were thermolabile and were destroyed soon after cell death), and (iii) MDV antigens were elaborated but were masked by production of an early antibody which attached to infected cells and blocked the subsequent attachment of fluorescing antibody. The first alternative seems unlikely in view of the positive correlation between antigen and pathology in most of the cases and the evidence presented showing that administration of specific antibody in infected chickens could block the pathological changes. The second explanation is illogical since foci of infection appear to spread from cell to cell, and most tissues should contain cells in various stages of infection. The positive FA tests in the tissues from other birds indicate no inherent deficiency associated with virus replication in the organs and tissues in question. The last possibility is worthy of consideration but requires more study. The PDRC strain of chickens employed for these studies is known to be immunologically very competent unless MDV infection is overwhelming (Calnek, Proc. Int. Symp.: Oncogenesis Herpesviruses Res., *in press*). Also, Chen and Purchase (6) demonstrated virus-associated antigens, on the surface of MDV-infected cells, which are reactive in the FA test.

The overall effect of the passive antibody was to reduce the level of, but not prevent, infection. In this respect, the situation bore a remarkable resemblance to that of canine herpesvirus infection of young dogs, in which passive antibody does not prevent infection but does prevent disease (4, 13). The low level of infection was evident not only from the depression of pathological response, but also because less viral antigen and fewer virus-infected cells were detected in birds with antibody than in those without antibody. Although many antibody-positive birds in experiment 1 were without evidence of infection at 7 to 14 days, examination at 28 days indicated birds from all groups to be positive; thus the antibody did not prevent infection but merely modified the level. Additional tests in experiments 2 and 3 confirmed that birds free of lesions or detectable viral antigen were indeed infected, although it was not actually established whether the effect was entirely one of preventing virus spread after infection. It could be that the antibody simply decreased the number of inoculum cells which successfully transferred virus to host cells, and thereby reduced the effective dosage. On the other hand, support of some efficacy of antibody against virus spread after infection was gained from the data from experiment 3 (Table 2), which showed a protective effect from antibody injected as late as 4 days after virus inoculation.

The mechanism by which circulating antibody might prevent spread of a cell-associated virus is not known. However, it has been clearly shown that cell-associated herpesvirus inocula induce fewer foci in cell cultures incorporating specific antibody than in cultures without antibody (8, 11; Calnek, *unpublished data*). Perhaps the antigens described by Chen and Purchase (5) attract antibody to the cell surface with the consequence of reduced efficiency of cell-to-cell virus spread.

The failure of HVT maternal antibody to protect chickens against the early pathological and virological effects of MDV infection is especially interesting in view of the use of HVT as a vaccine against MD in commercial chickens. The mechanism by which HVT is protective is unknown.

The widespread infection involving various visceral organs, as well as the hematopoietic organs, was of interest from a comparative standpoint, since similarly severe necrotizing infections may occur with herpesviruses in other species when young hosts are infected in the absence of maternal antibody (4, 23).

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